A P P L I C A T I O N

for

UNITED STATES LETTERS PATENT

on

SUPPRESSION OF CELL PROLIFERATION BY DECORIN

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EXHIBIT A

This invention relates to cell biology and more specifically to the control of cell proliferation.

Under normal circumstances, cell proliferation is a tightly controlled process; fast proliferation is needed during embryonal development and tissue regeneration, whereas the proliferation must be halted in the completed tissue. Cell proliferation appears to be controlled primarily by growth factors. Most of the known growth factors are stimulatory. Examples include epidermal growth factor, platelet-derived growth factor, various interleukins and colony-stimulating factors. A few negative regulators of cell proliferation are also known. Transforming growth factor beta is a multifunctional factor that inhibits the growth of some cell types, but can also stimulate proliferation. Other growth inhibitors include various interferons and a growth inhibitory role has also been ascribed to heparin, heparan sulfate and their fragments.

A less well understood mechanism of growth control relates to the close apposition of cells. Normal cells stop growing when they make contact with one another. This phenomenon, commonly known as contact inhibition of growth, is of obvious importance for the formation of orderly tissue structure.

A number of important pathological conditions depend on abnormal cell proliferation. The foremost of such conditions is, of course, cancer. Other diseases with a proliferative component include rheumatoid arthritis with its overgrowth of the synovial tissue, glomerulonephritis, in which the mesangial cells proliferate, and atherosclerosis, in which the abnormally proliferating cells are smooth muscle cells.

It is obvious from these examples that there is a great

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need to develop new methods for controlling cell proliferation. The present invention addresses this need and provides other related advantages as well.

SUMMARY OF THE INVENTION

The present invention relates to the proteoglycan Decorin (also known as PG-II or PG-40). The invention provides cells transfected with and expressing the gene coding for Decorin, and recombinant Decorin produced thereby. Spent culture media from such transfected cell cultures can be used to suppress the proliferation of either normal or abnormal cells. Moreover, purified Decorin can be used to suppress cell proliferation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the expression of Decorin in unamplified and amplified transfectants.

FIGURE 2 is a radiogram showing the expression of Decorin core protein in CHO cells.

FIGURE 3 shows morphological changes caused by expression of Decorin in CHO cells.

FIGURE 4 is a graph showing the growth of Decorin-expressing and control CHO cells in culture.

FIGURE 5 is a photomicrograph showing the effect of spent culture media on the morphology of CHO cells.

FIGURE 6 is a photomicrograph showing the effect of spent culture media on the morphology of Harvey ras gene-transformed NIH 3T3 cells.

FIGURE 7 shows the elution pattern from DEAE-Sepharose of spent culture media from Decorin-expressing cell line, clone 61.

DETAILED DESCRIPTION OF THE INVENTION

This invention stems from work performed to explore the functions of a proteoglycan, Decorin. Proteoglycans are proteins that carry one or more glycosaminoglycan chains. The known proteoglycans carry out a variety of functions and are found in a variety of cellular locations. Many of them, however, are components of extracellular matrix, where they participate in the assembly of cells to the matrix and affect the attachment of cells to the matrix.

Decorin, also known as PG-II or PG-40, is a small proteoglycan produced by fibroblasts. Its core protein has a molecular weight of about 40,000 daltons. The core has been sequenced (Krusius and Ruoslahti, Proc. Natl. Acad. Sci. USA 83:7683 (1986); Day et al. Biochem. J. 248:801 (1987), both of which are incorporated herein by reference) and it is known to carry a single glycosaminoglycan chain of a chondroitin sulfate/dermatan sulfate type (Pearson, et al., J. Biol. Chem. 258:15101 (1983), which is incorporated herein by reference). The only previously known function for Decorin is its binding to type I and type II collagen and the effect it has on the fibril formation by this collagen (Vogel, et al., Biochem. J. 223:587 (1984)).

A molecular biological study of Decorin has now led to

unexpected observations on its role in the control of cell proliferation, and these observations form the basis of this invention.

Decorin cDNA is transfected into cells, such as Chinese hamster ovary (CHO) cells, preferably those which are dihydrofolate reductase (dhfr)-negative, although other cells such as 3T3 and COS cells can also be used. Such transfection is accomplished by methods well-known in the art. The transfected cells are then grown in culture.

Chinese hamster ovary (CHO) cells into which human Decorin cDNA was transfected and which express the proteoglycan from this cDNA appear more adhesive to the substratum than the original cells. Moreover, the growth of the cells that expressed Decorin from the cDNA was suppressed and they grew to a lower saturation density than the various control cells. These controls included cells transfected with a construct expressing the core protein of Decorin and amplified to the same degree as the Decorin expressing cells. These cells were similar to the original CHO cells. The magnitude of the growth and adhesion changes was proportional to the amount of Decorin produced.

Moreover, changes in the adhesion and the saturation density could be reproduced with the spent culture media of the cells expressing the recombinant Decorin and with the Decorin isolated and purified from such culture media. These findings indicate that Decorin plays a previously unsuspected role in the control of cell proliferation, and that it can be used to modulate cell proliferation. The effect seen with oncogene-transformed 3T3 cells suggests that this invention may be useful in the treatment of proliferative diseases.

As used herein "Decorin" referes to a proteoglycan

having the structural characteristics attributed to it in Krusius and Ruoslahti, supra, and which suppresses cell proliferation as determined by the method of Example III. Human fibroblast Decorin has substantially the amino acid sequence presented in Krusius and Ruoslahti supra, figure 2, which is incorporated herein by reference. "Decorin" refers both to the native composition and to modifications thereof which retain the functional characteristics.

The recombinant Decorin of the invention has a structure corresponding substantially to that of the native proteoglycan. It is understood however that limited modifications may be made however without destroying the Decorin activity.

EXAMPLE I

EXPRESSION OF DECORIN AND DECORIN CORE PROTEIN

The 1.8 kb full-length Decorin cDNA described in Krusius and Ruoslahti, Proc. Natl. Acad. Sci. USA 83:7683 (1986), which is incorporated herein by reference, was used for the construction of Decorin expression vectors. For the expression of Decorin core protein, a mutagenized cDNA in which the fourth codon, TCT coding for serine, was changed to ACT coding for threonine was engineered by site-directed mutagenesis according to the method of Kunkel, Proc. Natl. Acad. Sci USA 82:488 (1985), which is incorporated herein by reference. The mammalian expression vectors pSV2-Decorin and pSV2-Decorin/CP (core protein) were constructed by ligating the Decorin cDNA or the mutagenized Decorin cDNA into 3.4 kb HindIII - Bam HI fragment of pSV2 (Mulligan and Berg, Science 209:1423 (1980) which is incorporated herein by reference), respectively. Dihydrofolate reductase (dhfr)-negative CHO

cells (CHO-DG44) were cotransfected with pSV2-Decorin or pSV2-Decorin/CP and pSV2dhfr by the calcium phosphate coprecipitation method. The transfected cells were cultured in nucleoside-minus alpha-modified minimal essential medium (α-MEM, GIBCO, Long Island) supplemented with 9% dialyzed fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Colonies arising from transfected cells were picked using cloning cylinders, expanded and checked for the expression of Decorin by immunoprecipitation from $^{35}SO_4$-labeled culture supernatants. Clones expressing a substantial amount of Decorin were then subjected to gene amplification by stepwise increasing concentration of methotrexate (MTX, Kaufman and Sharp, J. Mol. Biol. 159:601 (1982) which is incorporated herein by reference) up to 0.64 μM. All the amplified cell lines were cloned either by limiting dilution or by picking single MTX resistant colonies. Stock cultures of these established cell lines were kept in MTX-containing medium. Before use in experiments, cells were subcultured in MTX-minus medium from stock cultures and passed at least once in this medium to eliminate the possible MTX effects. Controls were transfected only with pSV2dhfr and treated exactly as experimental cells thereafter. Metabolic labeling of the cells with $^{35}SO_4$ or ^{3}H-leucine and immunoprecipitation was performed as described Brennan et al., J. Biol. Chem 259:13742 (1984), which in incorporated herein by reference.

Figure 1 shows the expression of Decorin in unamplified and amplified transfectants, by using fluorography of SDS-7%-polyacrylamide gel electrophoresis under reducing conditions. (A) $^{35}SO_4$-labeled culture supernatants immunoprecipitated with rabbit antipeptide antiserum prepared against the NH_2-terminus of human Decorin (Krusius and Ruoslahti, supra.). (B) Total $^{35}SO_4$-labeled products secreted into culture medium. (C) Total ^{3}H-leucine labeled products secreted into

culture medium. Lane 1: control transfectant A, an unamplified clone transfected with pSV2dhfr; lane 2: control transfectant C, a clone amplified to 0.64 λM MTX resistance from control transfectant A; lane 3: clone 1, an unamplified primary transfectant expressing 0.2 pg/cell/day of Decorin; lane 4: clone 31, a clone amplified to 0.32 λM MTX resistance and expressing 4 pg/cell/day of Decorin; lane 5: clone 61, a clone amplified to 0.64 λM MTX resistance and expressing 25 pg/cell/day of Decorin.

Figure 2 shows expression of Decorin core protein in CHO cells. Lanes 1 and 2: ^{3}H-leucine-labeled culture supernatants were immunoprecipitated as described in Figure 1. Lanes 3 and 4: Total ^{3}H-leucine-labeled products secreted into culture medium. Lanes 1 and 3: CHO cells transfected with pSV2-Decorin/CP. Lanes 2 and 4: Control CHO cells transfected with pSV2dhfr.

EXAMPLE II

QUANTITATION OF CELL SPREADING AND SATURATION DENSITY

The cell lines of Example I were plated in 24 well plates in MTX-minus culture medium at a density of $3x10^{5}$ cells per well. After 24 hours, medium was replaced (0.3 ml per well) and cells were incubated another 24 hours. Concentration of Decorin in these culture supernatants was determined by competitive ELISA (Engvall, Meth. Enzymol. 70:419 (1980) which is incorporated herein by reference). Briefly, a mixture of culture supernatant and rabbit antipeptide antibody against Decorin was incubated in the wells of microtiter plates coated with Decorin purified from human fetal membranes (Brennan et al., supra.). The amount of antibody bound to the wells was determined by alkaline-phosphatase-conjugated goat anti-rabbit IgG as a second

antibody. Various concentrations of purified Decorin were used to generate a standard curve. The cells were counted by hemocytometer at the end of the 24 hour incubation.

As shown in Table I, cells transfected with the Decorin gene exhibited a larger area of spreading than did control cells. Where Decorin expression was amplified, area of spreading increased with increasing expression.

Also shown in Table I are the saturation densities of the Decorin-expressing and control cells. In order to determine the saturation densities, cells ($1.2x10^5$) were plated in a 60 mm culture dish in MTX-minus culture medium. After 6 hours, cells were fixed with 3% paraformaldehyde and stained with toluidine blue. Quantitative evaluation of spreading was performed by measuring the surface area of the cells with a surface integration program of an image analyzer (Olympus). Nonspread cells were excluded from the measurement. The mean and standard deviation of values from 50 cells are shown.

TABLE I

PRODUCTION OF DECORIN AND SPREADING OF TRANSFECTANTS

Clone	Transfection	MTX Resistance (μM)	Decorin Prod. (μg per 10^6 cells/day)	Spread Area (μM^2/cell)	Saturation Density ($\times 10^{-5}$)
control line A	pSV2dhfr	0	0	2725 ± 627	10.8 ± 1.2
control line B	pSV2dhfr	0.32	0	2585 ± 693	10.4 ± 2.5
control line C	pSV2dhfr	0.64	0	2659 ± 586	10.6 ± 1.8
clone 1	pSV2-decorin + pSV2dhfr	0	0.2	3368 ± 842	9.9 ± 1.6
clone 31	pSV2-decorin + pSV2dhfr	0.32	4	4759 ± 898	7.3 ± 0.2
clone 33	pSV2-decorin + pSV2dhfr	0.32	11	5554 ± 1002	5.2 ± 0.2
clone 66	pSV2-decorin + pSV2dhfr	0.64	14	5482 ± 1382	4.9 ± 0.3
clone 61	pSV2-decorin + pSV2dhfr	0.64	25	6472 ± 1147	4.4 ± 0.4

EXAMPLE III

ANALYSIS OF THE EFFECT OF SPENT CULTURE MEDIA

The effect of spent culture media on the morphology of CHO cells and Harvey ras gene-transformed NIH 3T3 cells was determined by plating CHO cells in 35 mm dishes at a density of 2×10^5 cells/dish in two-day spent media from clone 61 containing approximately 20 μg/ml of Decorin and in similar media from control cell line C containing no Decorin and

cultured, the cell lines being those described in Example I.

Figure 5 shows the morphology of the CHO cells after this treatment and Figure 6 shows the morphology of the treated oncogen-transformed 3T3 cells. As can be seen, the spent culture medium from the Decorin-expressing cell line, clone 31, induced a morphology similar to that observed in the Decorin-expressing cells themselves. The oncogene-transformed 3T3 cells treated in this manner assume a morphology closely similar to that of normal cells. This morphology is often referred to as "contact inhibited morphology" and it is considered to be indicative of normal growth control. In accordance with this phenomenon, fewer cells were seen in these cultures compared to the control-media treated cultures. These results indicate that the culture media from the cell lines expressing Decorin reproduces the morphological and growth inhibiting effects seen in the recombinant Decorin-expressing cells themselves.

EXAMPLE IV

PURIFICATION OF DECORIN FROM SPENT CULTURE MEDIA

Clone 61 cells were grown to 90% confluence in 8 175 cm^2 culture flasks in nucleoside minus α-MEM supplemented with 9% dialyzed fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. At 90% confluence culture media was changed to 25 ml per flask of nucleoside-free α-MEM supplemented with 6% dialyzed fetal calf serum which had been passed through a DEAE Sepharose Fast Flow column (Pharmacia) equilibrated with 0.25 M NaCl in 0.05 M phosphate buffer, pH 7.4. Cells were cultured for 3 days, spent media was collected and immediately made to 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 0.04 mg/ml aprotinin and 5 mM EDTA.

Four hundred milliliters of the spent media were first passed through gelatin-Sepharose to remove fibronectin and materials which would bind to Sepharose. The flow-through fraction was then mixed with DEAE-Sepharose preequilibriated in 50 mM Tris/HCl, pH 7.4, plus 0.2 M NaCl and batch absorbed overnight at 4° C with gentle mixing. The slurry was poured into a 1.6 x 24 cm column, washed extensively with 50 mM Tris/HCl, pH 7.4, containing 0.2 M NaCl and eluted with 0.2 M - 0.8 M linear gradient of NaCl in 50 mM Tris/HCl, pH 7.4. Decorin concentration was determined by competitive ELISA as described above.

Figure 7 shows the elution pattern in DEAE-Sepharose Fast Flow. As can be seen, Decorin separates from the bulk of the protein present in the media and can be recovered in substantially pure form from the fractions showing the highest immune reactivity.

Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We Claim:

1. Cells transfected with Decorin cDNA.

2. Recombinant Decorin produced by the cells of claim 1.

3. Substantially purified recombinant Decorin.

4. A method of suppressing proliferation of cells by contacting said cells with spent culture media from Decorin-producing cells.

5. The method of claim 4 wherein said Decorin-producing cells are the cells of claim 1.

6. A method of suppressing proliferation of cells by contacting said cells with substantially purified Decorin.

7. The method of claim 6 wherein said purified Decorin is produced by recombinant means.

EXHIBIT A

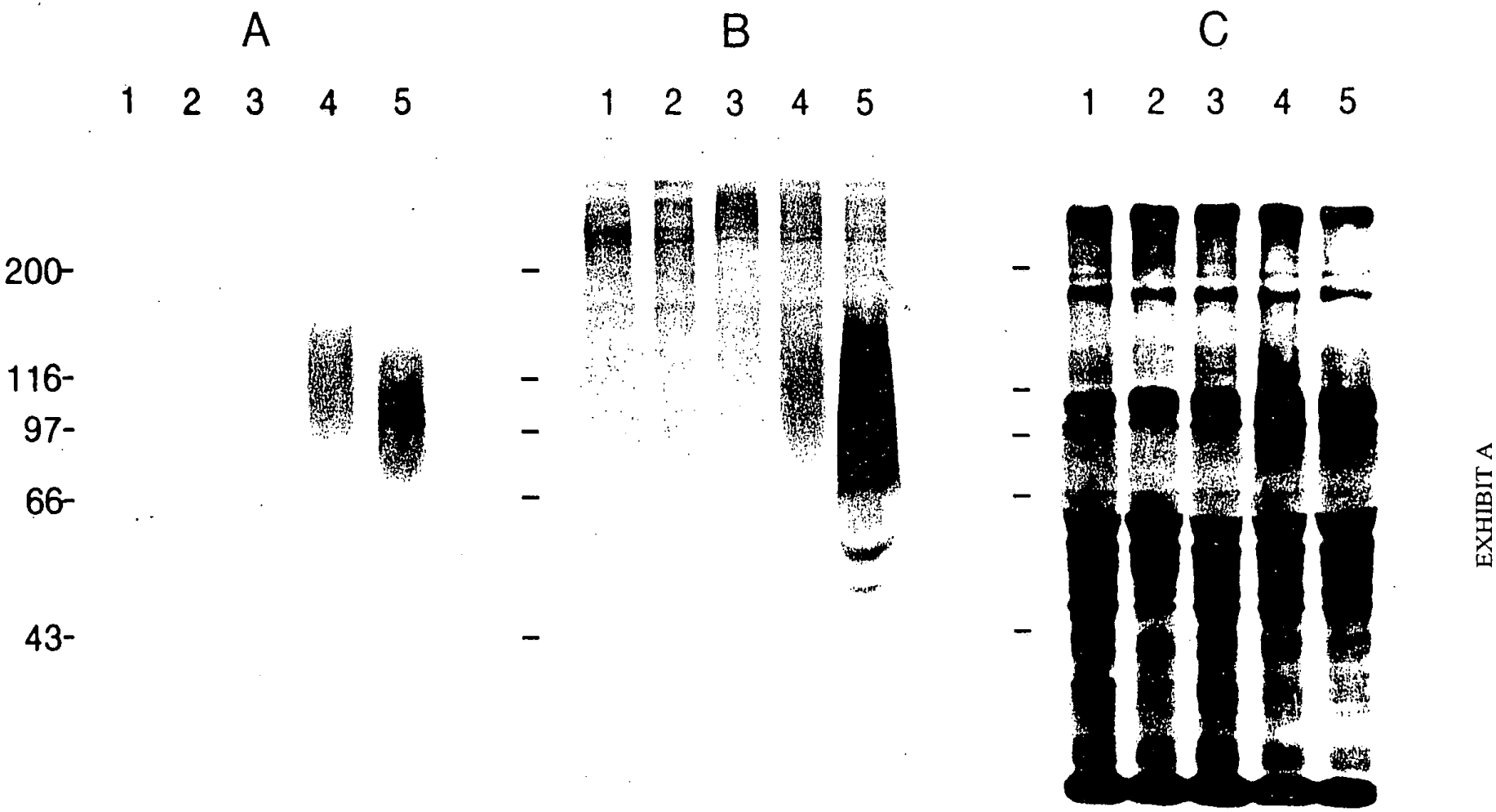
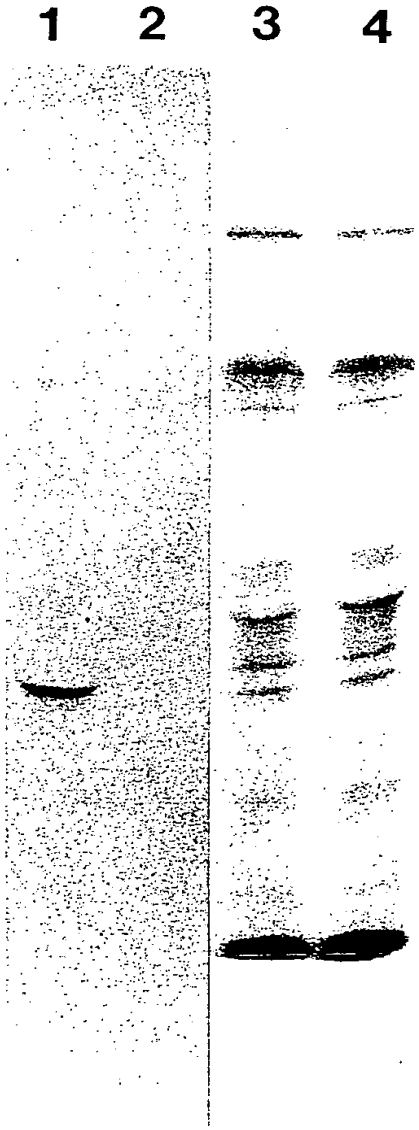


EXHIBIT A

FIGURE 1

FIGURE 2



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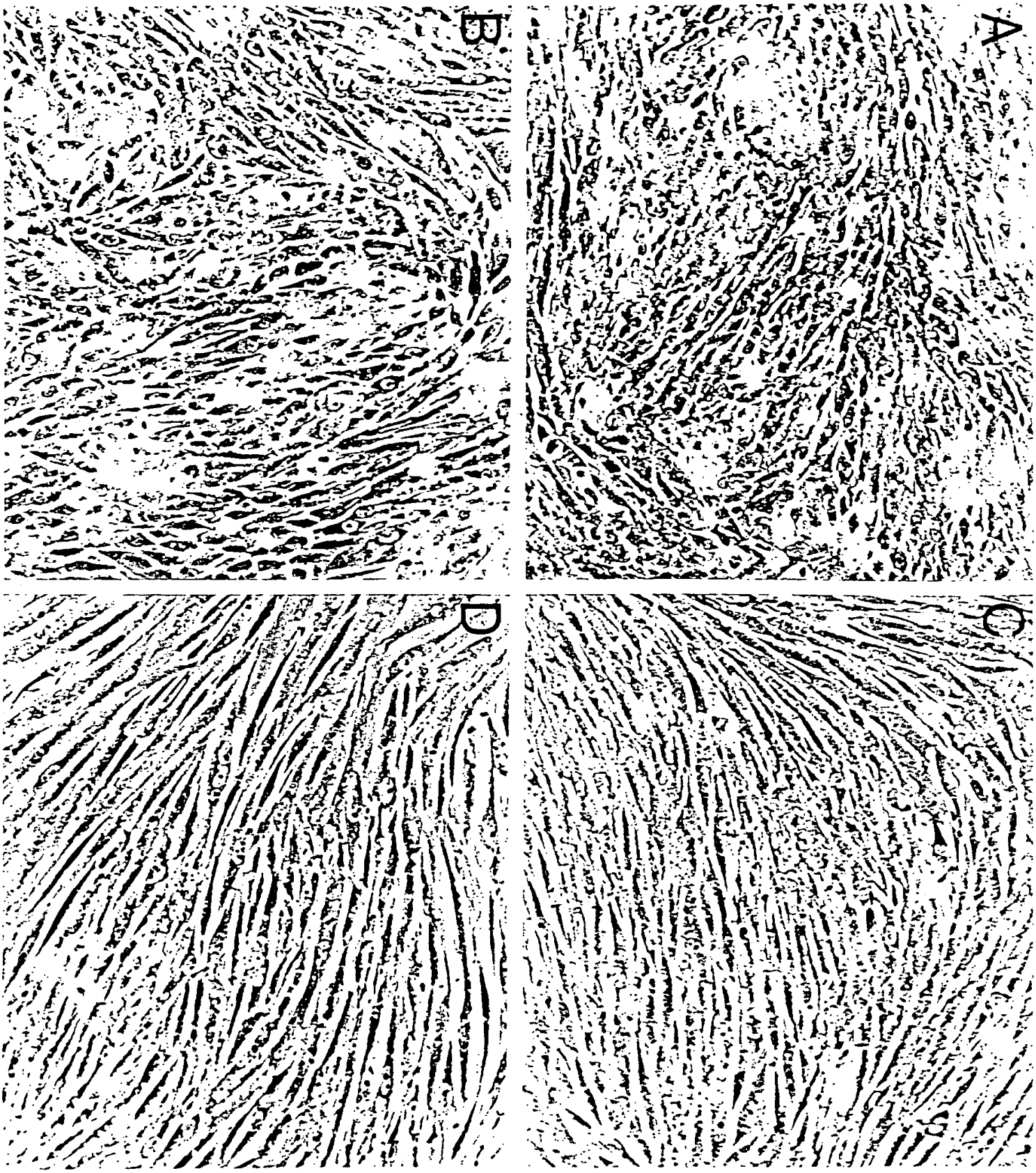

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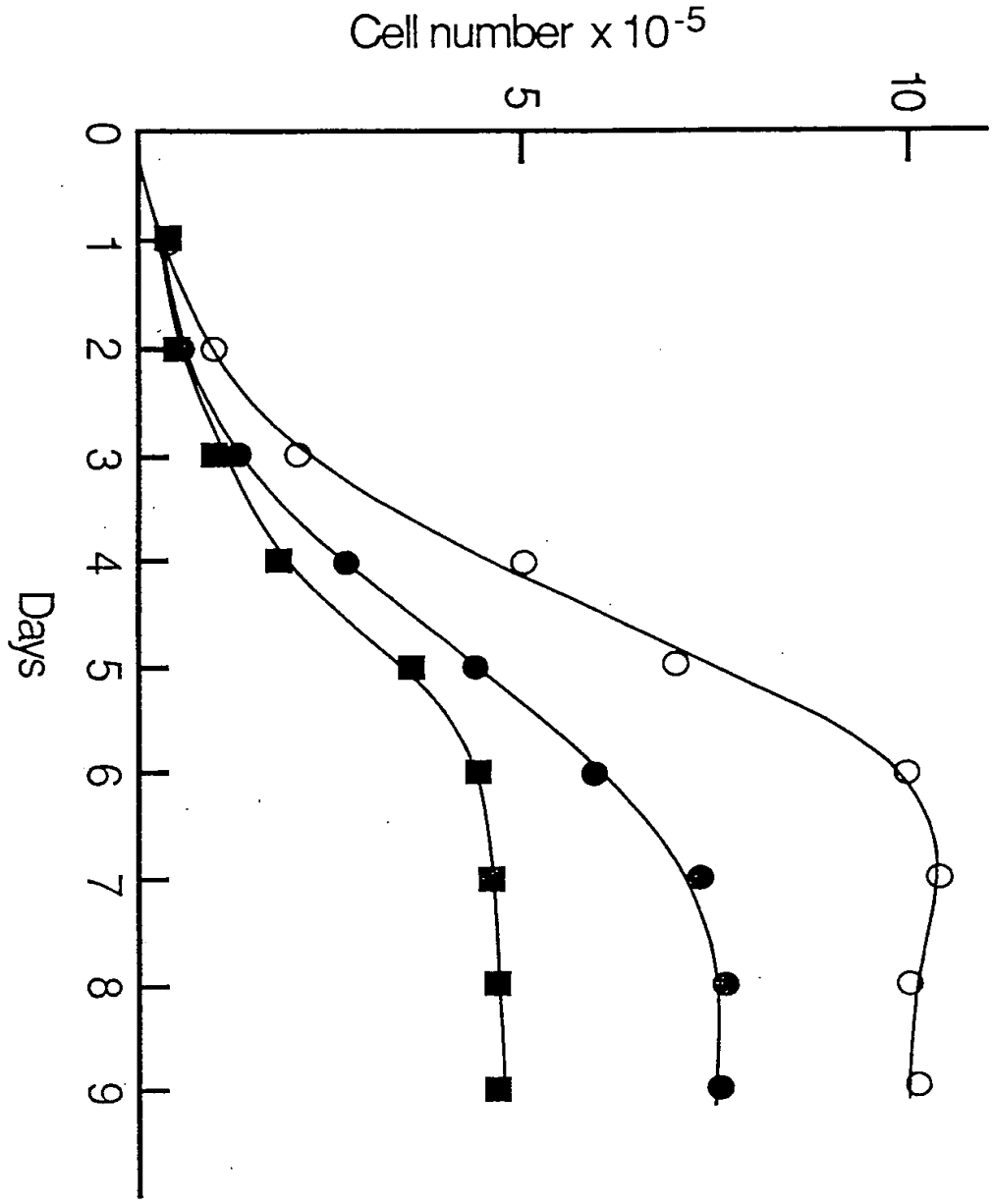

FIGURE 4

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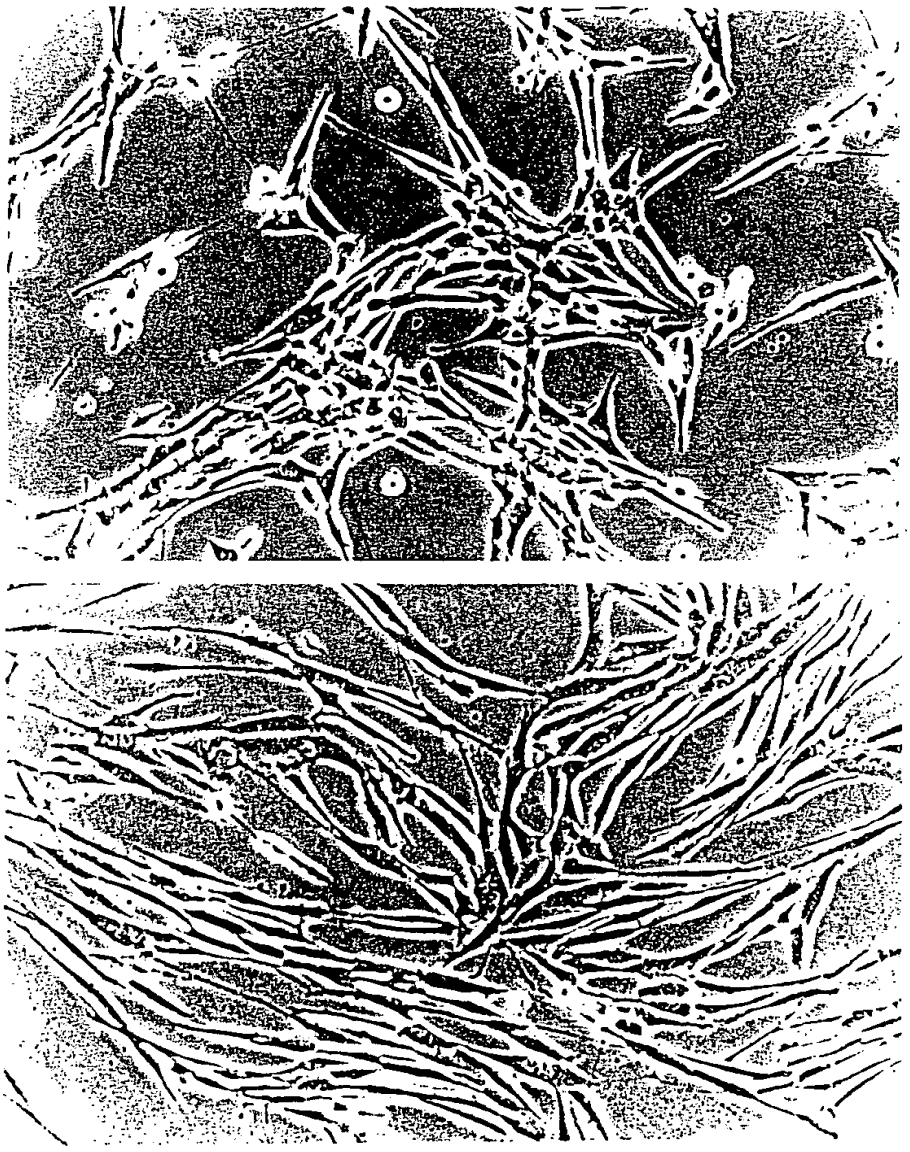

FIGURE 5

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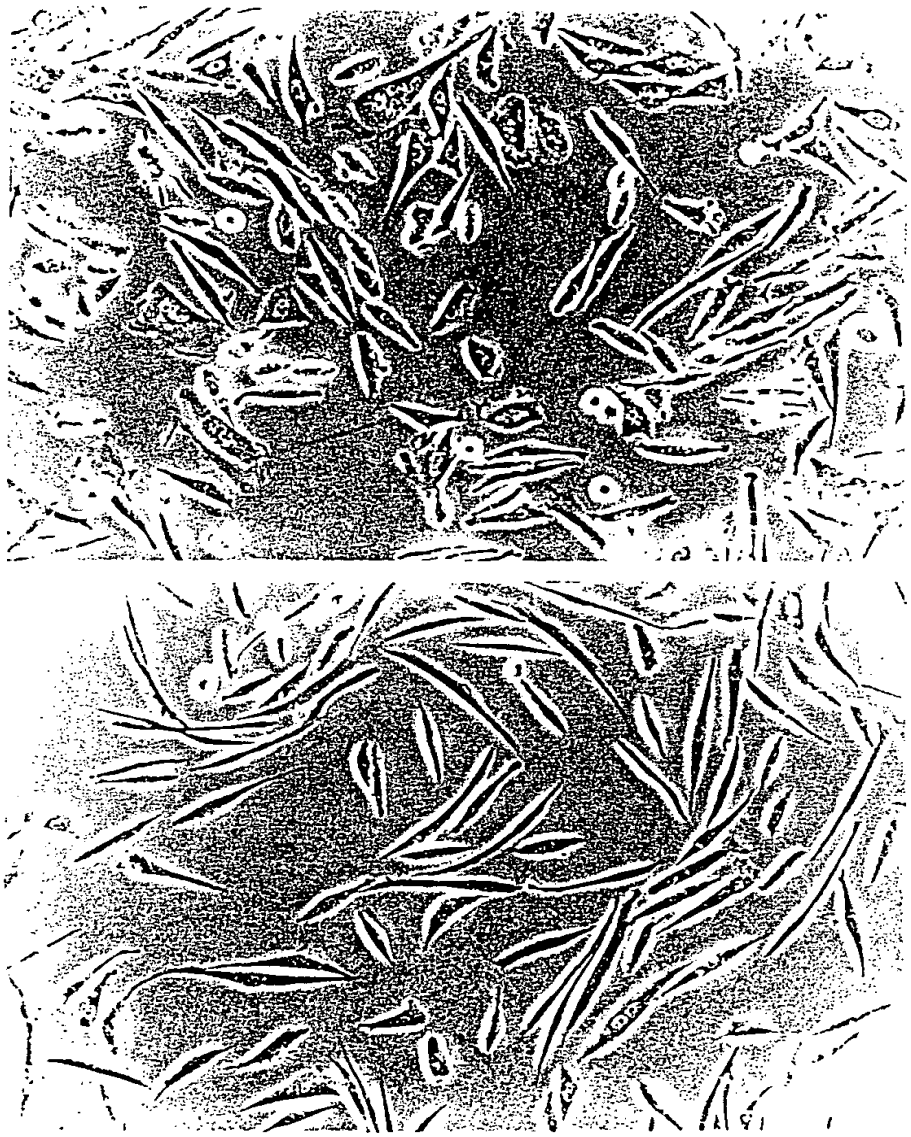

FIGURE 6

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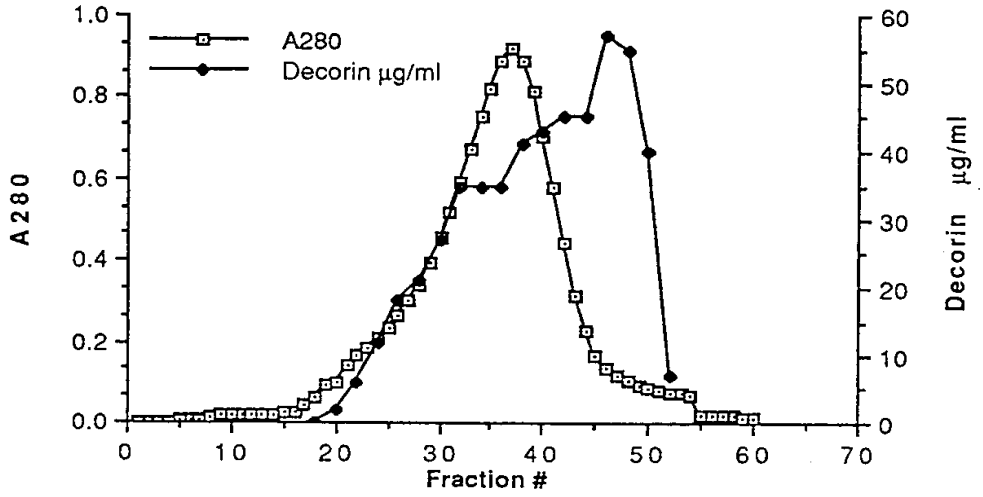

FIGURE 7

EXHIBIT A